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THE EFFECT OF RIBONUCLEASE DIGESTS OF AMINO ACYL-SRNA ON A PROTEIN SYNTHESIS SYSTEM

by Mituru Takanami

Space Sciences Laboratory, University of California, Berkeley

UNPUBLISHED PRELIMINARY DATA

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The biological synthesis of proteins is commonly believed to take place by the formation of polypeptide chains, so that the first amino acid in the chain is joined through its carboxyl group to the amino group of the next, proceeding sequentially until the terminal amino acid, which retains a free carboxyl group, is reached. This process takes place on ribosomes, and the polypeptide chain has a peptidyl-sRNA group attached to its growing end, and bound to the ribosome, at any stage of the synthesis.[1-5] The next amino acid is added to the growing polypeptide chain by coming to the adjacent site on the ribosome. A peptide bond is now formed between the amino group of this next amino acid and the terminal carboxyl group of the growing polypeptide chain, which is simultaneously liberated from esterification with the ribosyl group of the terminal adenosyl of sRNA. When puromycin is added to an amino acid incorporating system, the puromycin molecule can substitute for the incoming AA-sRNA, forming peptidyl-puromycin.[7-8] As the result, growth of the peptide chain ceases and the incomplete chain is released into the soluble phase.

In the present investigation it was found that T1-ribonuclease (T1-RNase) digests of AA-sRNA would bring about the release of incomplete polypeptide chains when added to cell-free preparations in which protein synthesis was in progress.

Such digests were presumed to contain oligonucleotide fragments some of which terminated with -CCA carrying an amino acid esterified to the terminal adenosyl group. Evidence was found suggesting that amino acids are transferred from such fragments to the C-terminal end of the released chain.

Materials and Methods--Ribosomes and Enzymes: The procedures for preparing ribosomes from E. coli strain B (H) were similar to those described previously.[9] The washed ribosomes were dissociated into subunits by dialysing against 0.25mM Mg^{++}-buffer solution, then recombined by raising the Mg concentration to 19 mM. The enzyme fraction was prepared as follows: the top 2/3 of the 100,000 x g 2 hour supernatant from the E. coli extract used in preparing the ribosomes was applied to a DEAE-cellulose column, and a fraction eluted from the column with 0.3M KCl was collected. The eluate was diluted and treated again with the DEAE-cellulose column. The 0.3M KCl eluate from the second column was used for incorporation of labeled amino acids into either sRNA or ribosomes. Such a fraction contains the mixture of enzymes necessary for incorporation of amino acids into polypeptide chains.[4]

AA-sRNA: E. coli B was grown in peptone broth[10] and sRNA was prepared from the cells by the method of Zubay.[10] The sRNA preparation was incubated with the enzyme fraction, ATP and amino acids under conditions optimal for amino-acid incorporation as determined separately.[4] After incubation at 37° C for 30 min., the re-

action mixture was chilled and shaken with an equal volume of 80% phenol. Two volumes of cold ethanol were added to the separated aqueous layer. The precipitate was dissolved in distilled water and 0.1 volume of 1M HCl was added. The precipitate formed was quickly collected, and dissolved by adding 1M acetate buffer, pH 5.8. Ethanol precipitation procedures were repeated twice. The final precipitate was dissolved, dialyzed against distilled water briefly, and used in experiments.

T1-RNase Digestion of AA-sRNA: T1-RNase was prepared from Taka-diastase (Sankyo Chem. Co., Tokyo, Japan) by the method of Takahashi.[11] The reaction mixture with sRNA (4 ml) contained 20 mg of sRNA, 200 moles of Tris-HCl, pH 7.0, and just sufficient T1-RNase, as determined separately by using graded amounts in a pilot experiment, to convert the sRNA added to an acid-soluble fraction during 10 min. at 37° C. Following this, the reaction mixture was chilled and shaken twice with cold phenol and the aqueous layer was separated. Phenol was then removed from the aqueous layer by shaking with ether. It was established by a separate experiment, using Sephadex (G25) column chromatography, that the amino acyl-adenylate bond was stable under the above conditions of digestion as reported elsewhere.[12,13]

Digestion with Pancreatic RNase: Conditions used for the digestion were similar to those described by Preiss et al.[14] The re-

action mixture (4 ml) contained about 20 mg of sRNA, 200 μmoles of acetate buffer, pH 5.8, and just sufficient RNase B (Worthington) to convert the sRNA added to an acid-soluble form in 10 min. at 30° C, as determined separately. The reaction mixture was incubated for 10 min. at 30° C, then treated with phenol, as described above.

Poly-UC was prepared from nucleoside diphosphates with Micrococcus lysodeikticus polynucleotide phosphorylase as described previously.[9] The base ratio of the product was found to be approximately 1U:2C.

Results and Discussion--Peptide Chain Synthesis in Poly-UC-Dependent Amino-Acid-Incorporation System: To detect the chain release in a normal incorporation system, ribosomes were incubated with enzymes, sRNA, energy system, and an amino acid mixture containing C^{14} serine, as a marker, in the presence of poly-UC (1:2) (see the legend to Fig. 1). At intervals indicated in the figure, aliquots of the reaction mixture were withdrawn, chilled, and centrifuged at 100,000 x g for 2 hours. The incorporation and distribution of the radioactivity were determined in the pellet and the supernatant and the results are in Fig. 1. Incorporation of amino acids reached a maximum within 40 min. Most of the radioactivity was found in the ribosome fraction. Some of the label seemed to transfer to the soluble phase with the incubation. However, it is difficult to evaluate this result, because prolonged incubation

also caused a decrease of the total label, perhaps due to the presence of E. coli proteases. When puromycin was added to a final concentration of 2×10^{-4} M over 60% of the radioactivity was released to the supernatant. (Fig. 1).

The Effect of RNase Digests of AA-sRNA On Nascent Peptide Chains: To make nascent peptide chain-ribosome complexes, a reaction mixture which had the composition shown in Fig. 1 was prepared and incubated for 20 min. at 37° C. Ribosomes were isolated from the reaction mixture by ultracentrifugation. Using these labeled ribosomes (about 2,400 cpm/mg), the effect of RNase digests was tested under the conditions described in the legend in Table 1. As a control, the effects of nontreated AA-sRNA and RNase digests of alkali-treated sRNA (30 min. at pH 10) were compared under similar conditions. As can be seen in Table 1, RNase digests of AA-sRNA, especially those of T1-RNase, caused the release of the label to the soluble fraction. Neither non-treated AA-sRNA nor RNase digests of alkali-treated sRNA showed a significant effect. It was also noted that RNAse itself had no effect.

Lagerkvist and Berg[15] found that T1-RNase digestion of sRNA produced fragments of eleven different sequences ranging in length from 4 to 9 nucleotides, including the -CCA terminal. The present investigation also indicated that amino-acyl-oligonucleotide fragments were present in the T1-RNase digest as determined by Sephadex

(G 25) column chromatography. On the other hand, treatment of sRNA with pancreatic RNase produces amino acyl-adenosine.[14] It seems possible that fragmental AA-nucleotides produced by RNase would replace normal AA-sRNA and incorporate in the C-terminal end of growing polypeptide chains to cause release of the chains, just as puromycin does.[8] The fact that the releasing effect of the T1-RNase digest was much more marked than that of pancreatic RNase seems to suggest that larger fragments produced by T1-RNase are much more efficient precursors for peptide bond formation than is amino acyl-adenosine. Further evidence is required for this conclusion, however, because the efficiency must also depend on the stability of these compounds in the reaction system, and because the effect with pancreatic RNase digests was small and variable.

Nathans and Neidle,[16] who compared the effect of amino acid analogues of puromycin, have reported that only aromatic amino acid analogues, such as those of phenylalanine, tryptophan and tyrosine (phe, try and tyr) were effective. Accordingly, the amino acids were stripped from sRNA and it was then charged either with aromatic (phe, tyr, try) and heterocyclic (histidine (his)) or non-aromatic amino acids, glycine, alanine, valine, leucine, serine and threonine (gly, ala, val, leu, ser and thr), treated with R1-RNase, and the effects were compared under similar condi-

tions. No marked differences between them were observed. (Fig. 2)

Transfer of Amino Acids from T1-RNase-Treated AA-sRNA to Peptide Chains: The following experiment was carried out to shed further light on the proposal that AA-nucleotide fragments incorporate into the C-terminal end of growing peptide chains and cause chain release: Ribosomes were incubated for 20 min. at 37° C in a reaction mixture containing poly-UC, sRNA, enzymes and a mixture of non-radioactive phe, ser, pro and leu, and were then isolated from the reaction mixture as in the previous experiments. In addition, strongly labeled sRNA was prepared using C^{14}-gly, -ala, -val, -thr, -aspartic acid (-asp)-glutamic acid (-glu) and -lysine (-lys), and was treated with T1-RNase. The digest was added to the above ribosome preparation, and incubated for 10 min. at 37° C. The incorporation and distribution of the radioactivity in both ribosomes and the supernatant were determined. This system contained only poly-UC as messenger RNA, and the reaction mixture of the second incubation did not contain any radioactive amino acid which should be coded by poly-UC (phe, leu, ser and pro). Nonetheless, significant transfer of amino acids from such AA-nucleotide fragments to the acid-insoluble fraction was observed; indeed, it was noted that over 75% of the radioactivity incorporated was found in the supernatant (Fig. 3). As a control, C^{14}-AA-sRNA, untreated with RNase, was incubated under similar conditions, and some incorpora-

tion was observed. In this case, however, the bulk of the radioactivity was found in the ribosome fraction. The incorporation in the second case was probably due to the presence of natural messenger RNA as a contaminant.

The supernatant fractions obtained by incubating ribosomes with the T1-RNase digest of the highly labeled AA-sRNA were pooled. The protein fraction was precipitated by adding trichloracetic acid (TCA), treated with hot TCA, then washed with ethanol to remove TCA. The washed protein fraction was dispersed in weak alkali (pH 9.0) and treated with carboxypeptidase (Sigma Chemical Co., DFP-treated). About 65% of the radioactivity was rendered soluble by digestion for 3 hours at 37^{o} C.

Discussion: In the cell-free system under investigation, we can postulate that sRNA molecules are retained on the ribosome on at least two sites. One of these is very likely the binding site discovered by Cannon et al.[17] who found one sRNA molecule firmly attached to a 50s subunit. The other site might be the messenger RNA site of the 30s subunit, because sRNA should first recognize the code of messenger RNA. If the base pairing between the coding triplet of messenger RNA and the corresponding complementing triplet of sRNA is involved in the interaction at this messenger RNA site, the binding energy would presumably be much weaker than that of the 50s site. In contrast, the binding energy at the 50s site

TABLE 1

Additions	Conc.	Enzymes*	Counts/min in supernatant
None	-	-	470
	-	+	740
Puromycin	2×10^{-4} M	-	4,450
AA-sRNA	"	-	690
	"	+	820
AA-sRNA, treated with RNase	"	-	2,750
	"	+	3,170
Alkali treated sRNA, treated with Tl-RNase	"	-	800
AA-sRNA, treated with P-RNase	"	-	1,050
	"	+	1,270
Alkali-treated sRNase, treated with pancreatic RNase	"	-	510
Pancreatic RNase	5 µg	-	640
	10 µg	-	720

* A mixture containing about 1 mg of the enzyme fraction (see Materials and Methods, paragraph 1), 2 µmoles ATP, 10 µmoles phosphoenol-pyruvate, 0.01 mg pyruvate kinase, and 0.1 µmole GTP.

should be strong enough to retain the whole sRNA molecule and also the growing peptide chain attached to its chain terminal. Under such circumstances, if an AA-nucleotide fragment comes to such a site, the present findings indicate that a peptide bond is formed. Such an amino acid would be accepted in peptide linkage without regard to the code at the messenger RNA site. Since the fragment is also presumably lacking or damaged in the binding site which normally attaches to the ribosome, the peptide chain would be released to the soluble phase.

In the present model of protein synthesis, no covalent bond is formed between the growing peptide chain and the ribosome, and the chain is retained on the ribosome through the terminal sRNA molecule alone. As shown in Table 1, however, pancreatic RNase even at a high concentration did not attack the terminal sRNA molecule attached to the ribosome. This would imply that the sRNA molecule, especially its -CCA terminal, is protected by contact, or stabilized by interacting either with the ribosome or with some protein, perhaps a transfer enzyme.

Summary: Preparations of aminoacyl-sRNA were digested with T1-ribonuclease to produce oligonucleotides with attached aminoacyl groups. Such digests were found to bring about the release of growing polypeptide chains when added to cell-free systems in which polypeptide synthesis was taking place, directed by poly-UC. This releasing effect is analogous to the action of puromycin, since

evidence was found that amino acids were transferred from the amino-acyl-oligonucleotides to the carboxyl-terminal end of the released chains.

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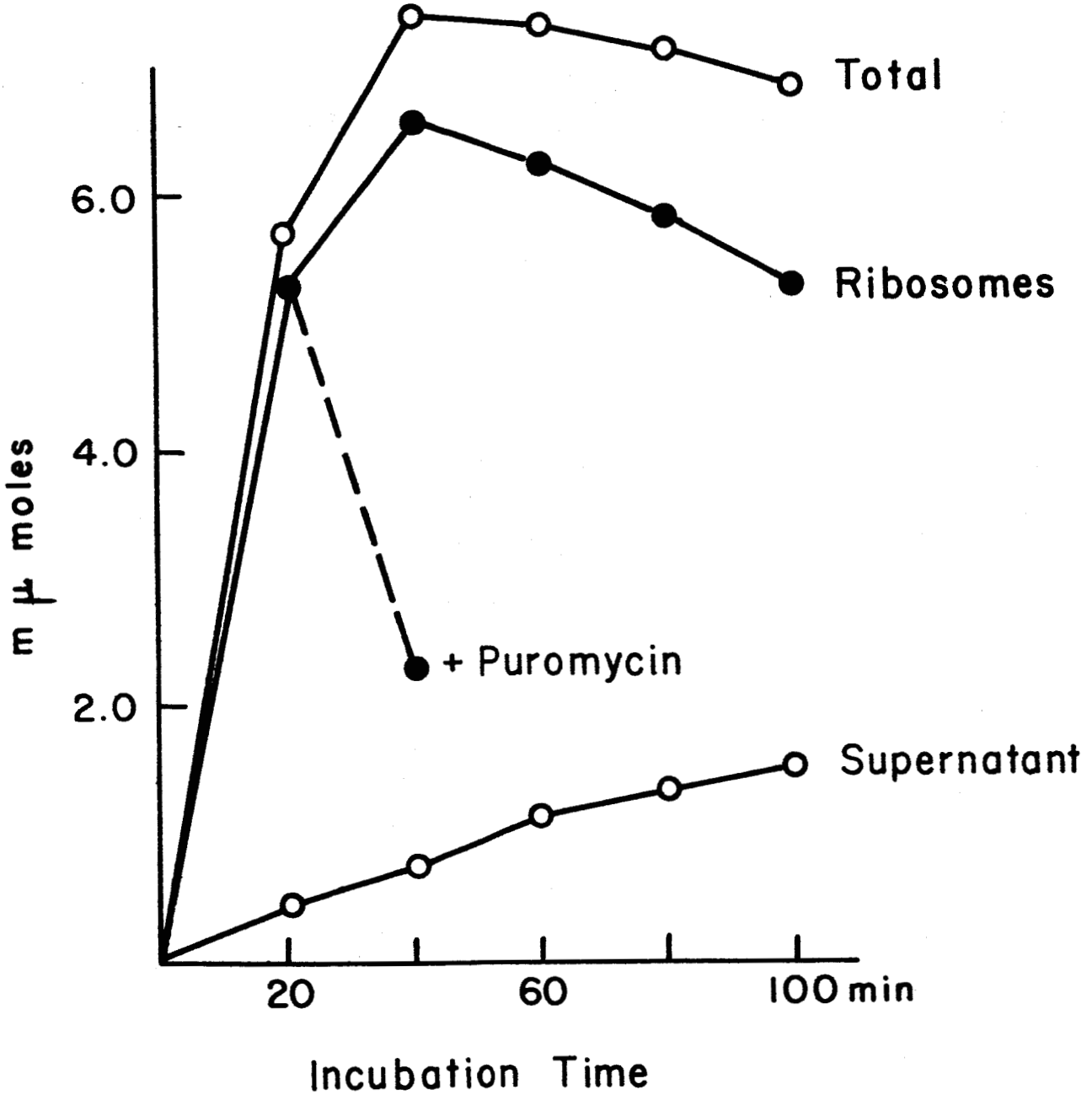
TABLE 1

THE EFFECT OF RNASE DIGESTS OF AA-SRNA ON THE NASCENT PEPTIDE CHAIN OF RIBOSOMES

A reaction mixture similar to that of Fig. 1 was prepared and incubated for 20 min at 37° C. After chilling, the reaction was centrifuged at 100,000 x g for 2 hours. The supernatant was removed and the pellet was resuspended in the buffer solution (10mM Mg^{++}, 5mM mercaptoethanol, 10 mM Tris-HCL, pH 7.6). The suspension was centrifuged for 20 min at 10,000 x g to remove insoluble materials. This fraction was designated as "labeled ribosomes".

The reaction system of the second incubation contained, in a final volume of 2 ml, 3 mg of labeled ribosomes (7,000 cpm), 120 µmoles KCL, 20 µmoles Mg^{++}, 100 µmoles Tris-HCL, pH 7.6, 0.2 µmoles spermine, and other additions as in Table 1. The reaction mixture was incubated for 10 min at 37°C. After chilling, 10 mg of carrier ribosomes was added and the mixture was centrifuged at 100,000 x g for 2 hours. Both the supernatant and the pellet were assayed.


Total
Ribosomes
+ Puromycin
Supernatant
6.0
4.0
2.0
mμ moles
20
60
100 min
Incubation Time

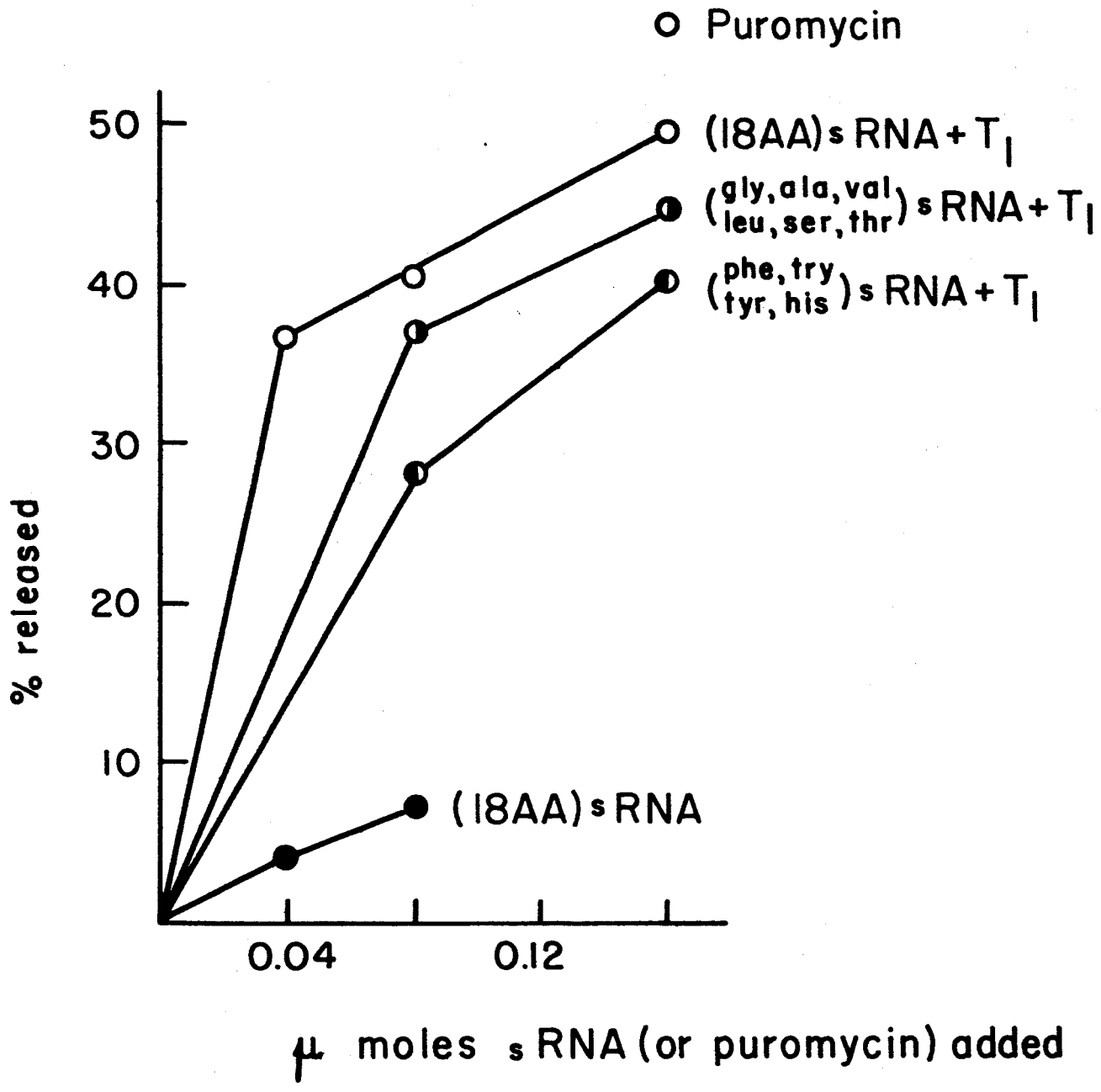

Puromycin
(18AA) s RNA + T1
(gly, ala, val / leu, ser, thr) s RNA + T1
(phe, try / tyr, his) s RNA + T1
(18AA) s RNA
% released
50
40
30
20
10
0.04
0.12
μ moles s RNA (or puromycin) added

FIG. 2.--Release of Nascent Peptide Chains from Ribosomes by T1-RNase Digests of Amino Acyl-sRNA.

The reaction system and the method of measuring the radioactivity were as in Table 1.

FIG. 1.--Poly-UC Dependent Amino Acid Incorporation

The reaction mixture contained, in a final volume of 20 ml, 40 mg ribosomes, 5 mg sRNA, (both determined as described previously[9]) 10 mg enzyme fraction, 20 μmoles ATP, 100 μmoles phosphoenol-pyruvate, 0.2 mg pyruvate kinase, 2 μmoles GTP, 2 μmoles spermine, 0.8 μmoles C^{14}-ser, and 2 mg of poly-UC(1:2). The reaction mixture was incubated at 37° C. At the intervals indicated, 2 ml of the reaction mixture was pipetted out, and chilled. About 10 mg of carrier ribosomes were added and the mixture was centrifuged for 2 hours at 100,000 x g. The top 2/3 of the supernatant was taken up. The pellet was washed once with the buffer solution. Both the pellet and aliquots of the supernatant were treated with hot TCA and assayed for radioactivity. The values for radioactivity in the supernatant represent TCA-insoluble material.

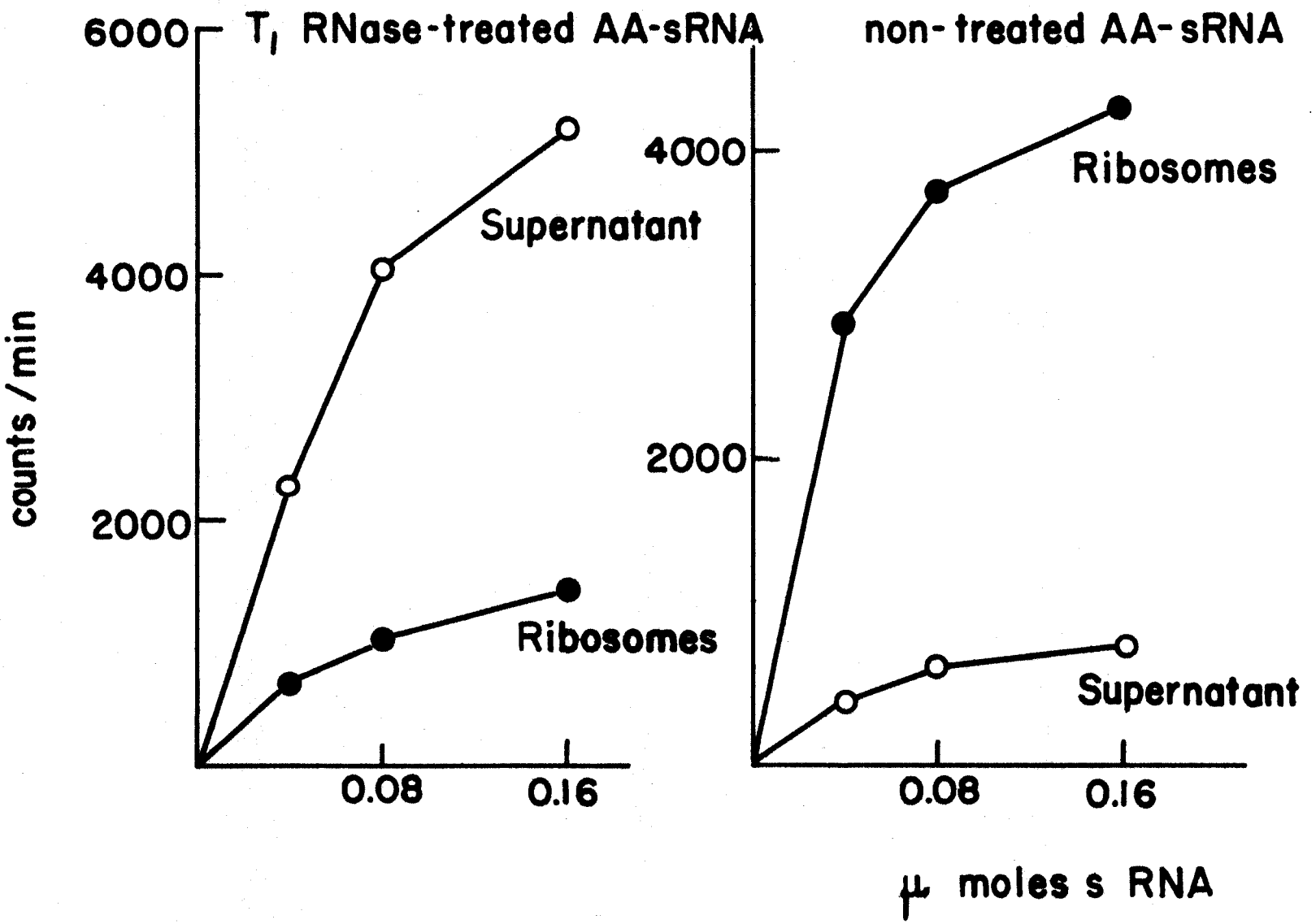

6000
4000
2000
counts / min
T1 RNase-treated AA-sRNA
Supernatant
Ribosomes
0.08
0.16
non-treated AA-sRNA
4000
2000
Ribosomes
Supernatant
0.08
0.16
μ moles s RNA

FIG. 3.--Transfer of C^{14}-amino acids from T1-RNase Treated sRNA to the Acid-Insoluble Fraction.

Ribosomes were incubated for 20 min. at 37° C in a reaction mixture, which was identical to that of Fig. 1, except for the composition of the added amino acids. In this experiment, 0.8 μmole each of non-radioactive ser, leu, phe, and pro were added. After incubation, the reaction mixture was centrifuged for 2 hours at 100,000 x g. The pellet was resuspended in the buffer solution. Insoluble materials were removed by low-speed centrifugation.

About 5 mg of these ribosomes were added to the reaction mixture (3 ml), containing 1 μmole GTP, 15 μmoles phosphoenolpyruvate, 0.03 mg pyruvate kinase, 0.3 μmoles spermine, 180 μmoles KCl, 30 μmoles Mg^{++}, 150 μmoles tris-HCl, pH 7.6 and T1-RNase digest of highly labeled C^{14}-sRNA (labeled with C^{14}-gly, ala, val, ser, thr, glu, asp, 60,000 cpm/mg). The reaction mixture was incubated for 10 min. at 37° C. After incubation, it was chilled, and centrifuged for 2 hours at 100,000 x g. Both the supernatant and pellet were assayed for radioactivity.